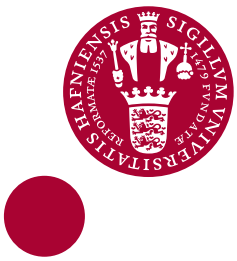

A cis-regulatory sequence acts as a repressor in the Arabidopsis thaliana sporophyte, but as an activator in pollen

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A *cis*-Regulatory Sequence Acts as a Repressor in the *Arabidopsis thaliana* Sporophyte but as an Activator in Pollen

Dear Editor,

Flowering plants undergo a life cycle in which the diploid sporophyte constitutes the predominant generation, and the haploid male and female gametophytes are reduced to only a few but highly specialized cells (McCormick, 1993). To achieve expression restricted to certain cell types, genes are not only actively expressed in these cells, they might also be repressed elsewhere. Here, we report that deleting an 88-base-pair (bp) promoter sequence of the pollen-specific gene *ACA7* redirects its expression to the sporophyte. Our study thus identified a bifunctional *cis*-regulatory module (CRM) that functions as a repressor in a plant sporophyte and as an activator in the male gametophyte.

Early mutation studies of pollen-specific promoters did not identify DNA sequences mediating gene repression (Twell et al., 1991; Eyal et al., 1995). Later, the *LAT59* gene of tomato was found to possess a sequence in its 5′ untranslated region that acts as a strong inhibitor of gene expression, probably by forming a stem-loop structure that causes proximal-promoter pausing of RNA polymerase (Curie and McCormick, 1997). However, the repressive function was exhibited in the sporophyte as well as in pollen (Curie and McCormick, 1997). Today, the only experimental evidence for a DNA motif conferring strict gene repression in every cell except the sperm cell in pollen is the 10-bp region GGCTGAATTT in the promoter of *LILY GENERATIVE CELL- SPECIFIC 1* (*LGC1*) in lily, which is recognized by the silencing transcription factor GERMLINE RESTRICTIVE SILENCING FACTOR (GRSF) in non-male germline cells (Haerizadeh et al., 2006). When the *GUS* reporter gene was expressed under control of the CaMV 35S promoter fused to the repressive GRSF binding motif, gene expression was abolished in transgenic *Arabidopsis thaliana* plants, suggesting the presence of a pathway to repress gene expression in the sporophyte (Haerizadeh et al., 2006). However, mutational studies of the pollen-specific *DUO POLLEN1* (*DUO1*) gene, which has a conserved GRSF binding motif in its promoter, found that *DUO1* expression is not regulated by this sequence; this implies that there must be other mechanisms for sporophytic repression of pollen-specific genes (Brownfield et al., 2009).

Expression of *ACA7*, which encodes for a plasma membrane Ca^{2+} P-type ATPase in *A. thaliana*, is restricted to pollen and its protein product is essential for proper pollen development (Lucca and León, 2012). To identify *cis*-regulatory elements involved in regulating the pollen-specific expression of *ACA7*, we created six constructs with *ACA7* promoter sequences of different lengths fused to the *beta-glucuronidase* (*GUS*) gene (Figure 1A, Supplemental Table 3 for primer sequences). These constructs were stably transformed into *A. thaliana* and *GUS* expression was analyzed by X-GlcA assay. The two constructs with the longest *ACA7* promoters (1504 bp and 1361 bp) gave rise to GUS activity in pollen (Figure 1B), which is in agreement with previous studies (Lucca and León, 2012) and microarray data (Supplemental Figure 1). Additionally, *GUS* was expressed in the tips of leaves, possibly hydathodes, which was not observed for endogenous *ACA7*, as revealed by reverse transcription PCR (Figure 1B and 1C). The pollen-specific pattern of GUS expression, however, was only observed in about 40% of individually transformed plants; a rate similar to that of a gene expressed in microspores and pollen (Honys et al., 2006). The remaining 60% showed expression in the vasculature of leaves and sepals, but not in pollen. With the third longest promoter truncation (1273 bp), surprisingly, 100% of individually transformed plants showed GUS activity in the vasculature of leaves and sepals, but not in pollen. This expression profile was also found in 100% of individually transformed plants with a much shorter promoter sequence of 429 bp. Truncating the promoter further (to 218 bp or 101 bp) resulted in a lack of GUS activity in all plant parts (Figure 1B). *GUS* expression was tested in T1 and T2 generations, and all results were identical across generations.

The results from the GUS expression analysis hinted at a regulatory element for pollen-specific expression located in the *ACA7* promoter between −1361 bp and −1273 bp from the start codon. The activity of the element may be influenced by genetic or epigenetic clues conferred by the transgene's insertion sites (Matzke and Matzke, 1998; Day et al., 2000), which would explain why, in independent transgenic lines including this element, stable expression was either in the gametophyte or in the sporophyte. In the absence of this element, expression was exclusively in the sporophyte depending on a promoter sequence further downstream (located between −429 bp and −218 bp from the start codon). Thus, the factor(s) that bind to the element required for pollen-specific expression probably establish repression by interacting with a sequence (or a factor binding to that sequence) several hundred base pairs apart. Taken together, our results indicate the presence of an upstream CRM with a dual function: activating gene expression in the male gametophyte and repressing it in the sporophyte.

Having identified an 88-bp CRM in the *ACA7* promoter, we searched the DNA sequence for the presence of known transcription factor binding sites. Using stringent search criteria, a DNA

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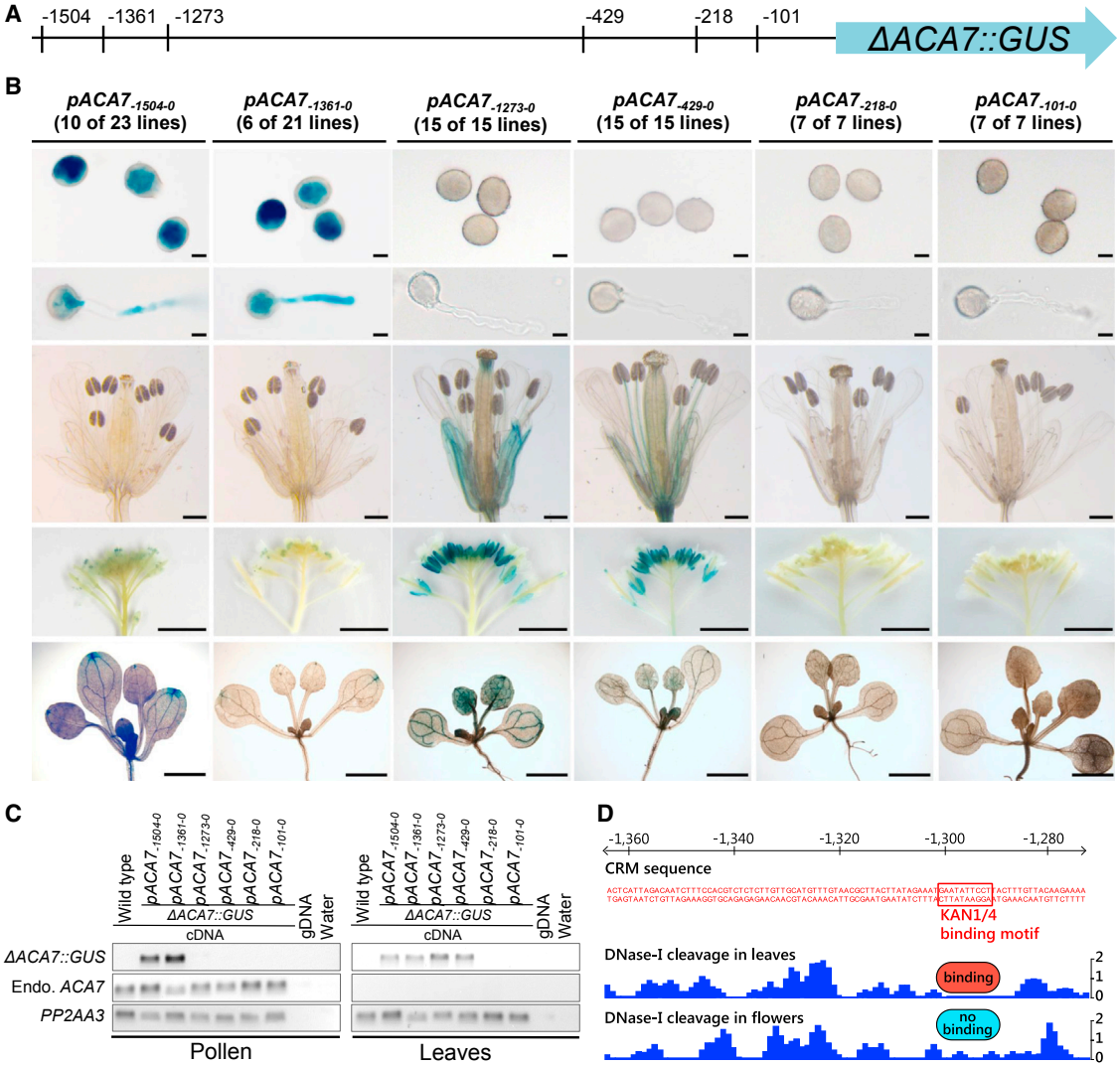

E

Analysis tool	TF binding motif	Number of motif occurrences[1] Seedlings	Pollen-specific
FIMO[2]	gaatat (KAN1/4)	0	0
SCOPE	gaatat (KAN1/4)	571	578

[1]within 1,000-bp promoter region; n = 541 for genes highly expressed in seedlings or genes with pollen-specific expression

[2]motif occurrences with a p-value < 0.0001

Figure 1. Pollen-Specific Expression of *ACA7* Is Established by a Bifunctional CRM in its Promoter.

(A) Schematic diagram showing the different lengths of *ACA7* promoter truncations. The first 71 codons of *ACA7* (*ΔACA7*) were fused to the *GUS* gene.

(B) *GUS* expression driven by *ACA7* promoter truncations of various lengths in stably transformed *A. thaliana* plants. The number of plant lines with a given expression pattern among the total number of screened lines is given under each construct. Expression in pollen and in tip of leaves can be seen for the longest promoter truncations fused to *ΔACA7::GUS* (*pACA7*$_{-1504-0}$ and *pACA7*$_{-1361-0}$). Shorter promoter lengths (*pACA7*$_{-1273-0}$ and *pACA7*$_{-429-0}$) confer *ΔACA7::GUS* expression in leaves and sepals, but not in pollen. No *ΔACA7::GUS* expression is observed for the shorter promoters (*pACA7*$_{-218-0}$ and *pACA7*$_{-101-0}$). Scale bars from top to bottom: 10 μm, 10 μm, 1 mm, 5 mm, and 2.5 mm.

(C) Reverse transcription PCR confirms the *ΔACA7::GUS* expression patterns observed in **(B)**. Endogenous *ACA7* (Endo. *ACA7*) is only expressed in pollen. *PP2AA3* serves as positive control for cDNA. Genomic DNA (gDNA) is from plants transformed with construct *pACA7*$_{-1504-0}$*::ΔACA7::GUS*.

(D) Detailed view of the CRM in the *ACA7* promoter. A potential transcription factor binding site (red box) is indicated. DNase cleavage of gDNA sampled in leaves and flowers of wild-type *A. thaliana*. In leaves, the potential transcription factor binding site is inaccessible to DNase-I, indicating protein binding. In flowers, which contain pollen, the site is less rigidly protected from DNase-I cleavage. Scale indicates normalized DNase-seq read counts.

(E) Occurrences of identified transcription factor binding motifs in promoter regions. Genes expressed in seedlings are compared with pollen-specific genes. No significant differences were found.

motif at position −1301 to −1291 was identified. The motif's sequence is GAATATTCCT and is recognized by KANADI4/ABERRANT TESTA SHAPE (KAN4, AT5G42630), which belongs to the *GARP* family of transcription factors. Another member of this family, KANADI1 (KAN1, AT5G16560), recognizes a part of the same motif (GAATAT) at position −1301 to −1295 (and its

palindromic sequence ATATTC at position −1299 to −1293). Both KAN1 and KAN4 are transcriptional repressors and are involved in determining the polarity of leaves (Huang et al., 2014). Noteworthy, the pollen-specific expression of the tobacco *NTP303* gene is driven by a *cis*-regulatory element with the sequence AAATGA (Weterings et al., 1995), which is present in an extension of the motif we found (AAATGAATATTCCT).

We tested if the identified DNA motif would be overrepresented in promoters of pollen-specific genes, compared with genes being highly expressed in the sporophyte, which we identified previously (Hoffmann and Palmgren, 2013). Using two different *in silico* tools to identify overrepresented DNA motifs, we did not find any differences in the abundance of these motifs within the different gene sets (Figure 1C, Supplemental Tables 1 and 2 show additional promoter lengths). We also analyzed the minimal *ACA7* promoter sequence necessary to drive *GUS* expression in the sporophyte (between 429 bp and 218 bp upstream of the start codon) for known transcription factor binding sites. No motif was found under the same stringent parameters as we used for analyzing the CRM (data not shown).

To further examine the biological relevance of the potential KAN1/4 binding site, we analyzed DNase-I cleavage data of genomic DNA purified from leaves and flowers (Zhang et al., 2012). The absence of DNase-I cleavage at a site indicates that proteins are binding to it, making the site inaccessible for DNase-I. We found that the DNA at the motif was inaccessible in leaves (Figure 1D). In flowers, however, DNase-I could cleave DNA at the motif, suggesting it was not (or less abundantly) bound by proteins. This finding indicates that the CRM is bound by proteins in leaves.

In summary, we have shown that an 88-bp DNA sequence forms a CRM that represses gene expression in the sporophyte, while activating it in pollen. This regulatory function must be driven by interaction of the CRM with other elements, of which one or more are located closer to the transcription start site. Additionally, because the activation in pollen and repression in leaves was found only in about 40% of individually transformed lines, the CRM's functionality may depend on genetic or epigenetic clues present at the transgene's insertion sites. Our finding of a bifunctional CRM is important for many aspects of gene regulation and the transcriptional changes underlying gametophyte development. Further experiments might identify the proteins involved in this process of gene regulation.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.D.H. and M.P.; Methodology, R.D.H., L.I.O., and M.P.; Investigation, R.D.H., L.I.O., J.O.H., J.S.N., J.F.B.T., A.P.W., and C.V.E.; Writing – Original Draft, R.D.H.; Writing – Review & Editing, R.D.H., L.I.O., and M.P.; Resources, M.P.; Project Administration, R.D.H.; Funding Acquisition, M.P.

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